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Research article

The effects of doxapram (blocker of K2p channels) on resting membrane potential and synaptic transmission at the *Drosophila* neuromuscular junction

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ABSTRACT

The resting membrane potential of most cells is maintained by potassium K2p channels. The pharmacological profile and distribution of various K2p channel subtypes in organisms are still being investigated. The *Drosophila* genome contains 11 subtypes; however, their function and expression profiles have not yet been determined. Doxapram is clinically used to enhance respiration in humans and blocks the acid-sensitive K2p TASK subtype in mammals. The resting membrane potential of larval *Drosophila* muscle and synaptic transmission at the neuromuscular junction are pH sensitive. The present study investigated the effects of doxapram on membrane potential and synaptic transmission using intracellular recordings of larval *Drosophila* muscles. Doxapram (1 mM and 10 mM) depolarizes the muscle and appears to depolarize motor neurons, causing an increase in the frequency of spontaneous quantal events and evoked excitatory junction potentials. Verapamil (1 and 10 mM) paralleled the action of doxapram. These changes were matched by an extracellular increase in KCl (50 mM) and blocked by Cd^{2+}. It is assumed that the motor nerve depolarizes to open voltage-gated Ca^{2+} channels in presynaptic nerve terminals because of exposure to doxapram. These findings are significant for building models to better understand the function of pharmacological agents that affect K2p channels and how K2p channels contribute to the physiology of tissues. *Drosophila* offers a genetically amenable model that can alter the tissue-specific expression of K2p channel subtypes to simulate known human diseases related to this family of channels.

1. Introduction

The resting membrane potential of cells is primarily driven by leakage channels that are selective for K^+ ions. This family of channels is generally referred to as K2p channels (two-P-domain K^+ subunits) (Buckingham et al., 2005; Plant and Goldstein, 2015). They were first identified in yeast and are known to be encoded in the genomes of yeast and humans (Goldstein et al., 1996, 1998).

Various subtypes of leakage channels are selectively expressed in different cells within an organism, which are selectively sensitive to volatile substances, free fatty acids, pH, membrane tension, hypoxia, heat, G protein-coupled receptor agonists, and other compounds (Enyedi and Czirják, 2010; Kim, 2005; Kamuene et al., 2021). Various diseases in humans are associated with dysfunctional K2p channels (Lee et al., 2021). There are 16 known types of K2p channels in humans, with varying pharmacological profiles and distributions in various tissues. Thus, while trying to target one tissue with an agonist or antagonist, other tissues are also impacted, leading to unwanted side effects by therapeutic treatments.

The pharmacology of the K2p channels varies among the subtypes, which can be an advantage while targeting selected subtypes in medical applications, such as induction of therapeutic hypothermia after an ischemic stroke or cardiac arrest (Komatsu et al., 2005; Yost, 2006; Song and Lyden, 2012) and treatments for respiratory disorders and apnea in infants (Cunningham et al., 2020; Vliegenthart et al., 2017). The use of therapeutic hypothermia may potentially improve the recovery outcome for cardiac arrest and hypoxia (Feketa and Marrelli, 2015; Chen et al., 2020). However, K2p channels are commonly targeted in this clinical treatment for desired outcomes.

To promote respiratory drive and reduce shivering with therapeutic hypothermia, doxapram (Stimulex or Respiram), an antagonist to K2p channels in the carotid bodies is used, which results in an increase in

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neural activity and thus respiration. The increased respiratory drive is likely due to the depolarization of these sensory cells. As expected, a number of associated side effects exist with the application of doxapram, as the subtype of K2p channels sensitive to doxapram may be expressed in many cell types (Baxter, 1976; Fathi et al., 2020; Lee et al., 2021). It is known that doxapram inhibits TASK, a K2p subtype in acid-sensitive mammals (Cotten et al., 2006; Kim, 2005). Thus, this type of K2p channel is expected to be present in carotid bodies and tissues, which show acid sensitivity related to changes in resting membrane potential.

The expression of these subtypes in various animal tissues is still being actively investigated. The subtypes of these K2P channels which maintain the resting membrane potential in skeletal muscle remain to be elucidated. Understanding the physiological role and examining the pharmacological actions of these potassium leak channels in different organisms and tissues will aid in better understanding their overall nature. *Drosophila* serves as a useful model to examine the function of many genes and proteins in a variety of physiological processes, such as muscle function, neurobiology, and synaptic transmission (Buckingham et al., 2005; Yamaguchi and Yoshida, 2018; Ugur et al., 2016).

The kcnk Ø gene codes for the K2p channel present in tissues obtained from neuromuscular junctions (NMJs). This tissue is composed of a variety of cell types but is predominantly muscle (Goldstein et al., 1996; Zilberberg et al., 2000, 2001; Ilan and Goldstein, 2001). This K2p channel is also referred to as the ORK channel in *Drosophila* (Buckingham et al., 2005). To date, there are 11 known kcnk Ø genes in *Drosophila* (Adams et al., 2000; Littleton and Ganetzky, 2000). The pharmacological and physiological profiles of K2p channel subtypes in *Drosophila* remain to be investigated (Buckingham et al., 2005). One of the ORK (or K2p) channels expressed in the pupal heart is known to promote a diastolic state and the tissue-specific knockdown promotes a faster heartbeat (Lalevee et al., 2006), while alterations in the CNS expression of K2p channels offset the circadian pattern in adult *Drosophila* (Park and Grith, 2006). The larval heart rate (de Castro et al., 2014) and membrane potential of body wall muscles (Badre et al., 2005) as well and synaptic transmission (Stanley and Cooper, 2021.) are highly

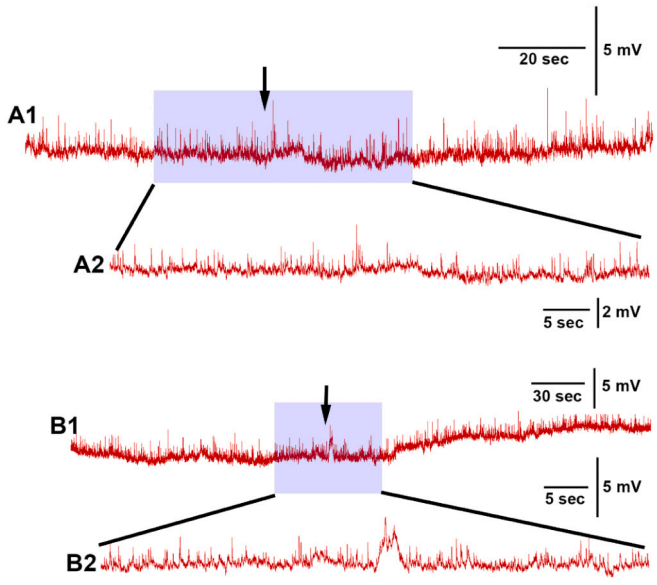

Fig. 1. Controls in exchanging the bathing media for compounds. Normal saline is exchanged for normal saline to account for disturbances in the preparation for exchanging the bathing media while recording membrane potential with an intracellular electrode in a muscle of larval *Drosophila.* Two representative trails are illustrated for two different preparations. (A) No change is present in membrane potential values upon changing the saline. (B) A slight deflection is present due to changing out the saline. Overall, no significant change in resting membrane occurs when the exchange is complete for the next 30 s.

pH-sensitive; therefore, it is likely that a K2p channel subtype that is known to be acid-sensitive, such as TASK, would be present in these tissues. Since doxapram targets this channel subtype in mammals, it was expected that this compound would potentially play a role in the motor nerve and body wall muscle in larval *Drosophila*.

Because there are known agonists and antagonists to various subtypes of K2p channels in mammals and K2p channels expressed in cell lines, these agents can be screened in functional assays using larval *Drosophila* to examine their effect on the resting membrane potential of muscle and the effects of associated motor neurons. For example, verapamil was recently shown to block the TRESK subtype of K2p channels (Park et al., 2018). Thus, same concentrations of doxapram and verapamil were used in the present study (1 mM and 10 mM).

The potential effects of alterations in synaptic transmission at the NMJs can serve as a window to the effects on neurons in *Drosophila*. In this study, we used doxapram as the compound, which is clinically used in human and veterinary care, to assess its effects on the resting membrane potential of larval body wall muscles, as well as to detect alterations in synaptic transmission at the NMJ.

2. Methods

2.1. Animals

Drosophila melanogaster Canton S (CS) flies were used in all behavioral and physiological assays. This strain has been isogenic in the laboratory for several years and was originally obtained from the Bloomington Drosophila Stock Center (BDSC). All the animals were maintained in vials partially filled with cornmeal-agar-dextrose-yeast medium.

2.2. Neuromuscular junctions of larval Drosophila

The effects of doxapram on the membrane potential, evoked excitatory junction potentials (EJPs), and spontaneous quantal events (mEJPs) were examined upon exposure. Third-instar *D. melanogaster* larvae were dissected in physiological saline (described in detail, Mattingly et al., 2018). The segmental nerves were cut and sucked into a suction electrode filled with saline and stimulated. Segmental nerves were stimulated at 0.5 Hz (S88 Stimulator, Astro-Med, Inc., Grass Co., West Warwick, RI, USA). To monitor the transmembrane potentials of the body wall muscle (m6) of 3rd instar larvae, a sharp intracellular electrode (30–40 M resistance) filled with 3 M KCl impaled the fiber. An Axoclamp 2 B amplifier (Molecular Devices, Sunnyvale, CA, USA) and a 1 × LU head stage were used. The use of ground lead in a 1 % agar plug within a 200 µl Eppendorf pipet tip, made with saline, reduced DC offset from varying saline levels on the ground lead within the recording dish when changing the media. Fly saline was used as previously described (de Castro et al., 2014). The preparation was then bathed in physiological saline: (NaCl 70 mM, KCl 5 mM, 20 mM $MgCl_2{\cdot}6H_2O$, $NaHCO_3$ 10 mM, Trehalose 5 mM, sucrose 115 mM, BES 25 mM, and 1 mM $CaCl_2{\cdot}2H_2O$; pH 7.1). Doxapram powder was added directly to saline to obtain 1 or 10 mM and placed on a vortex (high setting) for 5 min. Doxapram saline solution remained opaque. Verapamil powder was readily dissolved in HL3 saline to obtain concentrations of 1 mM and 10 mM.

High concentrations of doxapram (1 and 10 mM) were used in these studies as this was a novel preparation to investigate. Based on previous studies a 100 µM produced maximal effect on mammalian nerve cells (Cunningham et al., 2020; Kruszynski et al., 2019); thus, a 10 times higher concentration was used herein. Given 1 mM did not produce the nerve to produce evoked events, a higher concentration was also used (10 mM). The concentrations of verapamil was used to mimic the concentrations of doxapram for direct comparisons. A 1 mM Cd^{2+} was used to block the presynaptic calcium channels in the nerve terminal (Potter et al., 2021). The duration of exposure to doxaparm and verapamil were

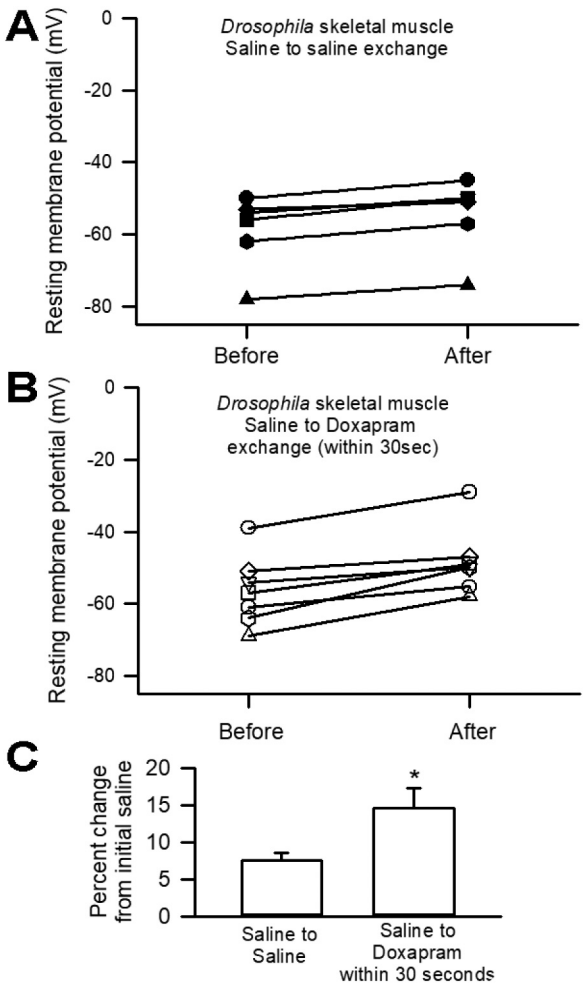

Fig. 2. The effects of doxapram exposure on resting membrane potential of *Drosophila* skeletal muscle. Each line in graphs A, B, and C represent individual preparations. (A) The saline-to-saline exchange serves as a control for the time factor while maintaining an intracellular recording. On average, a slight depolarization occurs over time. (B) The effects of doxapram exposure on resting membrane potential of *Drosophila* skeletal muscle within 30 s is shown. (C) A percent change from the mean of the initial saline to the exchanged media is shown. A significance in the change of membrane potential is present within 30 s for doxapram exposure as compared to saline ($P < 0.05$ *t*-test).

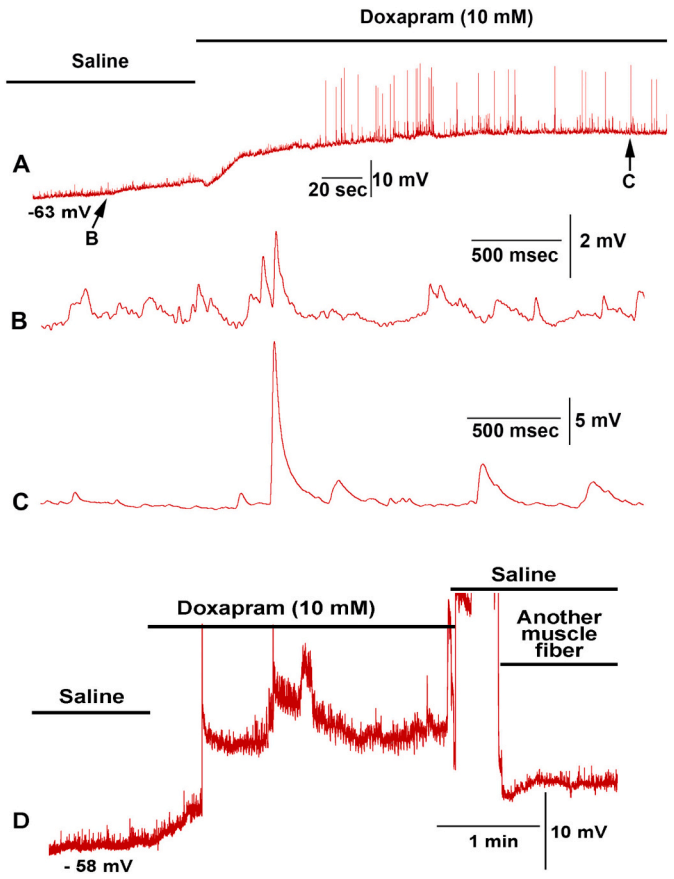

Fig. 3. The effect of exposure to doxapram (10 mM) on the membrane potential and synaptic transmission at the larval *Drosophila* neuromuscular junction. (A) The membrane depolarizes rapidly, and spontaneously evoked excitatory junction potentials (EJPs) occur upon exposure to doxapram. (B) As demarcated in A with the arrow and letter, an enlarged section of the trace reveals miniature excitatory junction potentials (mEJPs) that are present in the absence of doxapram. (C) An enlarged section of the trace shown in A, as highlighted by the arrow and letter, illustrates the large EJP and mEJPs with doxapram exposure. (D) Another preparation exposed to 10 mM doxapram illustrating the differences with some preparations showing a rapid effect and strong contractions. After losing the intracellular recording for the preparation shown in D another m6 muscle in the adjoining segment was recorded to test for viability of the preparation. The same trends were present in 6 of 6 trials (Wilcoxon rank sum test $P < 0.05$).

as long as the recordings were suitable, usually until the contractions of the muscle fiber were so large that an intracellular recording was not feasible due to the damage of the fiber. The KCl concentration of 50 mM was determined by trial and error by how high of a concentration was needed to induce what appears as evoked EJPs by the motor neuron as was induced by 10 mM doxapram. After each compound at least the bath was exchanged two times with fresh saline, in some cases three times to ensure the bathing solution with the compounds were removed. The bath exchange is a delicate procedure as not to dislodge the intracellular recordings.

2.3. Statistical methods

In general, Shapiro–Wilk tests were used to check for the normality of the data and to determine the use of a *t*-test or Wilcoxon signed rank test.

3. Results

The resting membrane potential of the m6 muscle was measured before and during exposure to doxapram. Exchanging saline while maintaining an intracellular recording can result in slight changes in the membrane potential due to disturbances in the preparation. Over time, the membrane potential depolarizes due to membrane injury while maintaining an intracellular recording. Control experiments were conducted to account for changes over time and to exchange the bathing media. Resting membrane potentials were obtained for the effect of doxapram within 30 s of exposure. The change in saline to saline as a sham control resulted in a change in the potential in some instances, which can be seen in the trace, while in other preparations, no deflection was observed (Fig. 1). The change in saline with one containing doxapram or verapamil at 10 mM showed a rapid (within 30 s) depolarization compared to saline-only exchanges. The percent change in the resting membrane was compared for normalization among preparations (Fig. 2A–C, $P < 0.05$ *t*-test).

3.1. Spontaneous quantal activity

In transected segmental nerves from the CNS, spontaneous quantal events (mEJPs) occur at a high frequency and dissipate over several

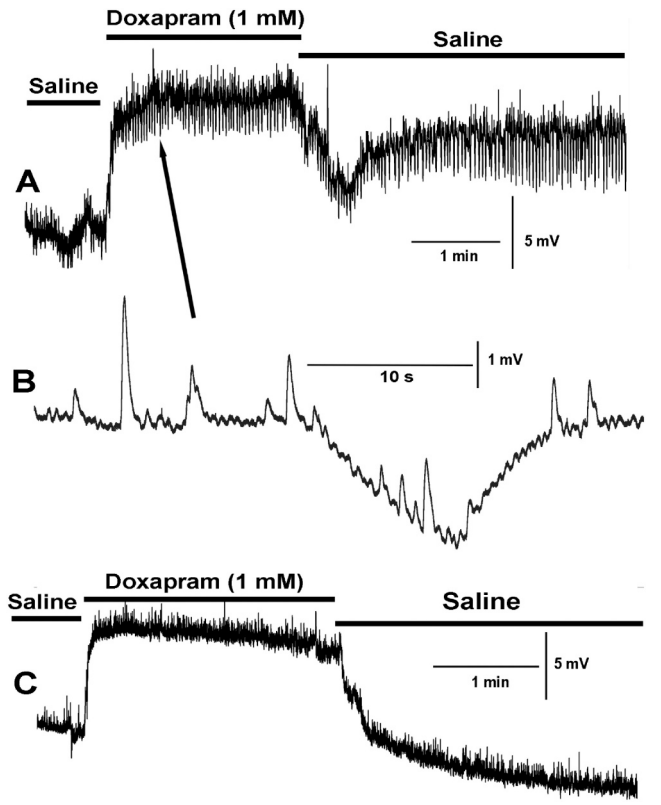

Fig. 4. The effect of exposure to doxapram (1 mM) on the membrane potential. (A) The membrane depolarizes rapidly, and spontaneously quantal excitatory junction potentials (mEJPs) occur upon exposure to doxapram. (B) As demarcated in A with the arrow and letter, an enlarged section of the trace reveals miniature excitatory junction potentials (mEJPs) that are present in the presence of doxapram. (C) Another preparation exposed to 1 mM doxapram illustrates the differences with some preparations. Here there are no waves of contractions to alter the membrane potential. The same trends in a small (~5 mV) depolarization were present in 6 of 6 trials (Wilcoxon rank sum test $P < 0.05$).

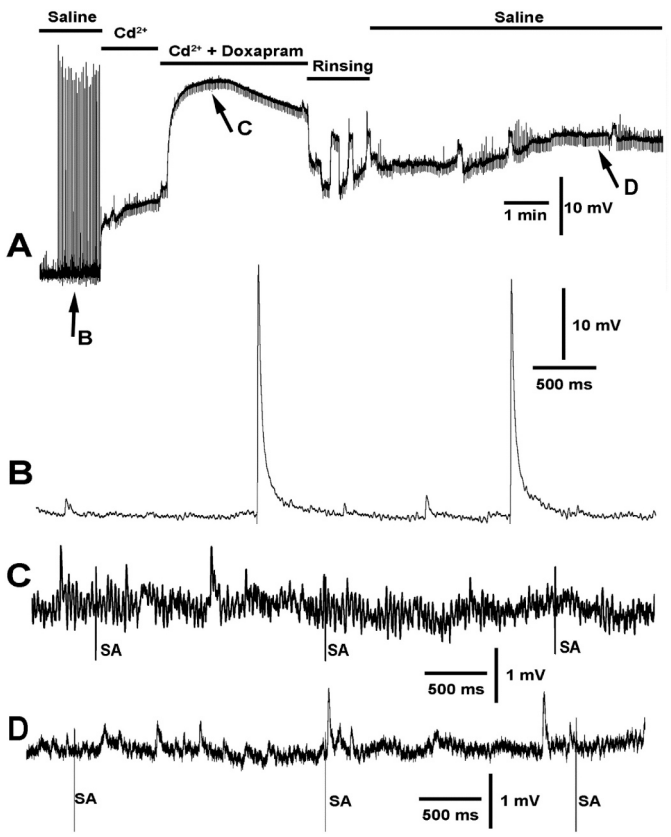

Fig. 5. The effect of exposure to doxapram in the presence of Cd^{2+}. Cd^{2+} was used to ensure blocking of presynaptic voltage gated Ca^{2+} channels as well as Ca^{2+}channels on the plasma membrane of the muscle. (A) Evoked excitatory junction potentials (EJPs) were rapidly reduced in amplitude upon exposure to Cd^{2+}. (B) Evoked excitatory junction potentials (EJPs) were present in saline solution. The segmental nerve was being electrically stimulated at 0.5 Hz. (C) During exposure to Cd^{2+} and doxapram (10 mM), no evoked generated EJPs were recorded but a few spontaneous quantal events can be observed. (D) After refreshing the bath with saline only the evoked responses are still depressed and only a few spontaneous quantal events appear. The same trends in Cd^{2+} blocking evoked transmission as well as doxapram exposure from inducing large EJP were present in 6 of 6 trials (Wilcoxon rank sum test $P < 0.05$).

minutes. Longer dissected preparations remain in the saline with transected segmental nerves, and the frequency of spontaneous quantal events dissipates (Lee et al., 2009). The frequency of spontaneous events rarely increases with time in saline. However, upon exposure to doxapram, the change in frequency was rapid, along with a slight depolarization of the muscle (Fig. 3).

The transected segmental motor nerves in normal physiological saline do not evoke events unless they are electrically stimulated. However, it appears that doxapram may induce depolarization of the motor neurons as large spontaneous EJPs occur (Fig. 3A,C and D). Owing to the immense increase in the frequency of mEJPs, it was difficult to count the number of mEJPs as they were superimposed on each other and produced broad depolarizations in addition to random EJPs. Fig. 3A–C and D show representative examples of maintaining an intracellular recording and exchanging the bathing media to one containing doxapram and the large non-stimulated EJPs. This trend was observed in each preparation recorded ($N = 6$; $p < 0.05$, rank-sum sign test). As shown in Fig. 3A, the muscle twitched so hard that it damaged the muscle fiber, and a saline washout was not feasible. The same was observed for the preparation shown in Fig. 3D; however, another fiber in the same preparation could be recorded. The fibers with an intracellular electrode in place when adding 10 mM doxapram were mostly damaged by the contractions, thus making it difficult to relay long-term recordings of the membrane potential.

A lower concentration of 1 mM doxapram would still result in small contractions, such as waves throughout the larval segments; however, some recordings could be maintained to assess the effects of flushing the preparation with fresh saline in an attempt to remove the doxapram (Fig. 4). Two different preparations have been illustrated. Three of the six preparations produced wave-like contractions and repeated downward deflections in membrane potential. However, the other three preparations did not show waves of contraction. In all six preparations, there was a large increase in the occurrence of spontaneous quantal events with exposure to doxaparm (1 mM), and the high rate of occurrence persisted even after the preparations were flushed with fresh saline without doxapram. This is likely due to depolarization of the presynaptic nerve terminal.

3.2. Evoked EJPs

In the presence of evoking EJPs at a frequency of 0.5 Hz before and during exposure to doxapram, the resting membrane potentials are more difficult to maintain due to muscle twitching from the spontaneous mEJPs along with the doxaparm-evoked EJPs.

To determine whether the increase in mEJPs and non-evoked EJPs was due to Ca^{2+} influx in the presynaptic terminal, saline containing Cd^{2+} (1 mM) was exchanged with the original saline and allowed to incubate while recording evoked EJPs. When the evoked EJP ceased to occur, because Cd^{2+} blocks presynaptic Ca^{2+} channels, the bath was

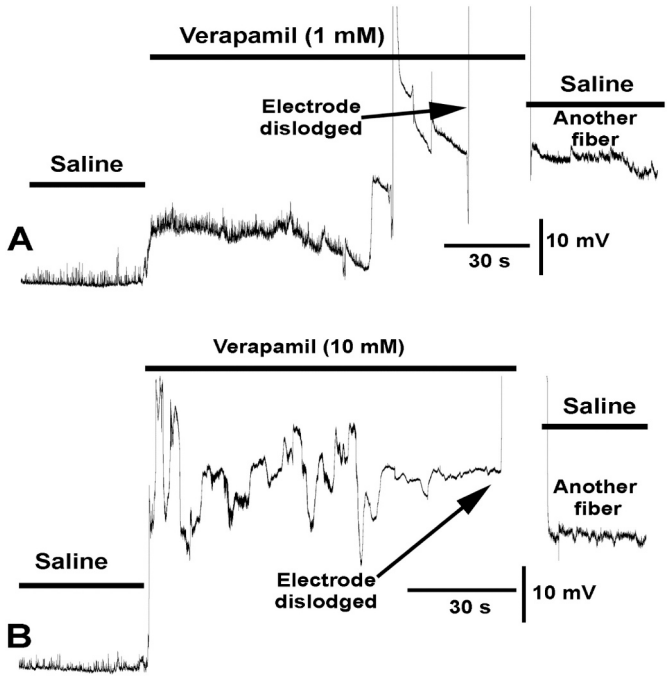

Fig. 6. The effect of exposure to verapamil on the membrane potential of the larval *Drosophila* muscle. (A) Exposure to 1 mM would rapidly depolarize the muscle and would lead to strong contractions in a short time ($N = 6$, Wilcoxon rank sum test $P < 0.05$). The fiber initially being recorded from would be damaged. So, after flushing the bath with fresh saline not containing verapamil, an analogous muscle either one segment removed or on the contralateral side would be recorded to examine viability after removing verapamil. (B) Exposure to 10 mM verapamil immediately produced strong contractions and would also result in losing the intracellular recording (N = 6, Wilcoxon rank sum test P < 0.05). After flushing the preparation with fresh saline and recording from naive muscle fibers, the resting membrane potentials were still in a depolarized state and would show small waves on contraction.

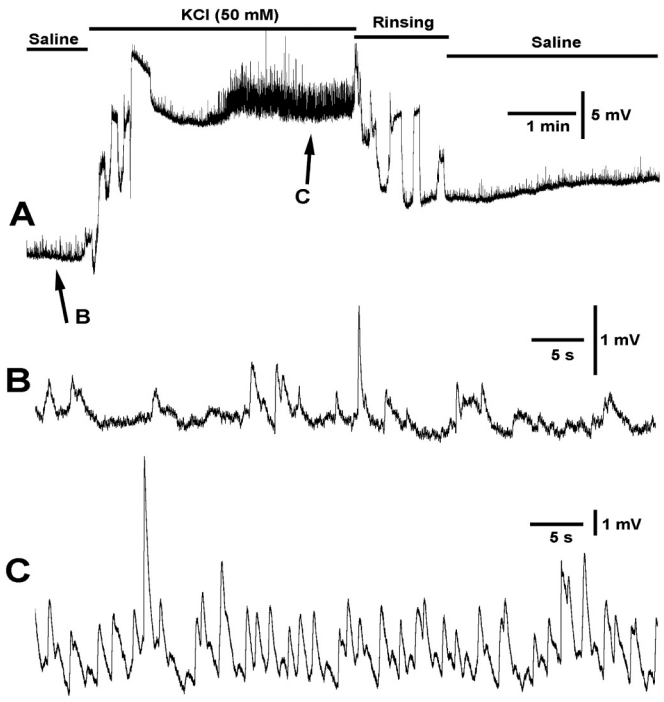

Fig. 7. The effect of exposure to raised KCl (50 mM) on synaptic transmission at larval *Drosophila* neuromuscular junction. (A) When exposed to raised KCl, the frequency of the mEJPs increased rapidly as well as random large EJPs which were presumed to be due to membrane depolarization of the motor nerve terminal. The rinsing stage shows the dislodging of the intracellular electrode for a short period due to muscle twitching. (B) The mEJPs are present in saline solution. (C) During exposure to KCl, the frequency of the mEJPs significantly increased. The same trends with raised extracellular K^+ were present in 6 of 6 trials (Wilcoxon rank sum test $P < 0.05$).

exchanged with saline containing doxapram (10 mM) and Cd^{2+} (1 mM). A large increase in the frequency of mEJPs that occurred when doxapram alone was applied was not as prevalent (Fig. 5). The evoked EJPs remained suppressed, indicating that presynaptic voltage-gated Ca^{2+} remained blocked by Cd^{2+}. No random large EJPs occurred, which would have been induced by the large influx of Ca^{2+} within the presynaptic nerve terminal. This trend was present in all the seven trials ($p < 0.05$, rank sum sign test, Fig. 5).

Since verapamil has been shown to block some types of K2p channels, the concentrations used was the same as doxapram to directly compare to the effects. In all the six cases for 1 mM and 10 mM, the contractions of the fibers were so large that intracellular recordings could not be maintained (Fig. 6). However, after exchanging the saline with fresh saline without verapamil and recording in a fiber not damaged, such as one on the contralateral side of the larvae but also an M6 fiber, a membrane potential can be obtained. The preparations would still have a contracted state if previously exposed to verapamil at 10 mM but would have a more relaxed state for the preparations previously exposed to 1 mM and rinsed with fresh saline.

3.3. Depolarization of the muscle with KCl

To examine whether depolarization of the motor nerve by doxapram was sufficient to induce an increase in spontaneous mEJPs and alter the induced EJPs, saline with an increased concentration of KCl (10 mM to 50 mM) was explored. Saline (50 mM) resulted in greater membrane depolarization of the muscle fibers than that observed with doxapram, but at this concentration, the motor nerve would depolarize enough to fire action potentials as seen when evoking EJPs by nerve stimulation (Fig. 7).

The frequency of mEJPs and alterations in the evoked EJPs were not as prevalent as for exposure to doxapram in the lower KCl concentration containing saline 10 mM and even 20 mM. The lack of bursting of mEJPs and no neural induced EJPs in lower than 50 mM extracellular K^+ indicates that doxapram has an equal to or greater effect than 50 mM extracellular K^+. The membrane depolarized by 14.5 ± 1.2 mV (mean ± SEM; $N = 7$) to a mean resting membrane potential of 42.0 ± 2.5 mV with exposure to 50 mM K^+. In all the seven preparations, the muscle would depolarize and appear to have stabilized, and within the three-minute observation period, the muscle would contract and dislodge the intracellular electrode.

4. Discussion

The present study demonstrated that doxapram depolarized the skeletal muscle in larval *Drosophila*, increased the frequency of mEJPS, and resulted in motor neurons spontaneously evoking EJPs. This may have occurred due to the presynaptic nerve terminal depolarizing and opening of voltage-gated Ca^{2+} channels as a result of blocking of the K^+ leak channels. This was implied because the effects on synaptic transmission could be inhibited by the presence of Cd^{2+}, which blocks voltage-gated Ca^{2+} channels, and because raising extracellular K^+ to 50 mM could induce similar effects as doxapram. Exposure to verapamil at the same concentrations as doxapram showed enhanced effects, leading to muscle contractions.

The subtypes of K2p channels vary along neurons and among cell types in organisms studied to date (Abdul Kadir et al., 2018; Fernández-Fernández et al., 2018; Medhurst et al., 2001; Feliciangeli et al., 2015).

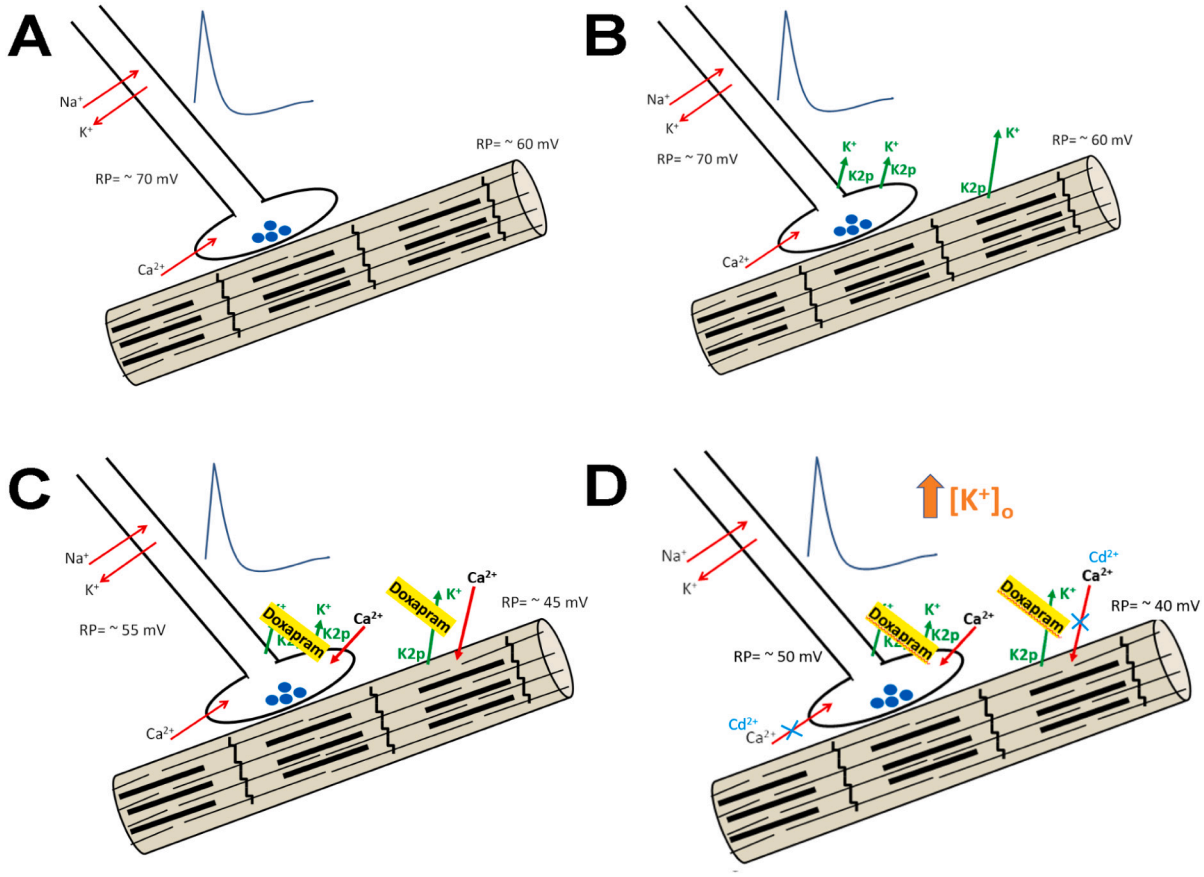

Fig. 8. A schematic model of *Drosophila* neuromuscular junction (NMJ) and the effects of the experimental paradigms used. (A) *Drosophila* NMJ model at rest. (B) *Drosophila* NMJ model at rest and highlighting K2P change location on both the muscle fiber and the motor neuron. (C) The *Drosophila* NMJ model upon doxapram exposure, demonstrating the blockage of the K2p channels and membrane depolarization. (D) With raised K^+, Cd^{2+} blocks the Ca^{2+} channels on both the NMJ and muscle fiber and doxapram blocks K2p channels, resulting in the membrane depolarization.

The density of subtypes in skeletal muscle is yet to be identified, and some K2p channels are known to have different distributions along the axons of neurons (Schwarz, 2021). Various subtypes of K2P channels are expressed in the central and peripheral nervous systems of mammals (Talley et al., 2001, 2003). Thus, it is likely that nerve terminals have a different distribution than the cell bodies or axons of motor neurons in *Drosophila*. The subtypes present in presynaptic motor nerve terminals have yet to be identified in *Drosophila* and mammals. Since the larval muscle did not depolarize to zero with exposure to droxapram, there are likely other non-doxapram sensitive K2p channels that contribute to membrane potential. This could also be the case for the motor neurons; however, this cannot be determined readily in larval *Drosophila*, as one cannot impale the axons of the motor neurons to directly measure the membrane potential.

The Ca^{2+} required for muscle contraction in larval *Drosophila* arises from extracellular Ca^{2+} as Ca^{2+} channels open on the plasma membrane due to depolarization. The presence of Cd^{2+} while exposed to doxapram prevented heavy twitching in the muscle, which was helpful in assessing the effect of doxapram on the resting membrane potential. When doxapram was applied without Cd^{2+}, the muscles demonstrated fasciculations, and it was difficult to maintain an intracellular recording owing to muscle contractions. One noted side effect of doxapram in human studies is fasciculation of the skeletal muscle, which is most likely due to enhanced motor nerve activity. However, this could also be due to the depolarization of the skeletal muscle to reach the threshold for action potentials in mammalian skeletal muscle.

Verapamil has been used on Drosophila larval muscle before and to separate out the two types of Ca^{2+} currents on larval muscle. Gielow et al. (1995) had shown one type of current is blocked by dihydropyridines and diltiazem. This is similar for vertebrate L-type Ca^{2+} channels. Another type of Ca^{2+} current was blocked by amiloride as for vertebrate T-type Ca^{2+} channels. It would be interesting to further investigate if doxapram has any action on these Ca^{2+} channels in larval *Drosophila* muscle in addition to the potential action on the K2p channels.

To highlight the experimental approach used and the potential actions of doxapram application and the role of the K2p channels in this *Drosophila* NMJ model, a schematic model of the NMJ is shown (Fig. 8).

In summary, the *Drosophila* model offers a possibility for pharmacological identification of the K2p subtypes and their role in the physiological aspects related to synaptic transmission at NMJs and in maintaining the resting membrane potential of muscle fibers. This was illustrated by the examination of verapamil in this study. Because transcription can be transiently regulated in the genetically amenable *Drosophila* model, one can acutely retard the normal synthesis of specific subtypes of K2p channels or even induce inserted genes for other subtypes in specific tissues to determine the functional properties of the various K2p subtypes in different organisms. Future studies are needed to address pharmacokinetics and dose-responses not only in *Drosophila* preparations but also in other experimental synaptic models to address the effects on synaptic transmission and neural activity.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

Data availability

Data will be made available on request.

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